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# Reassessing the ichthyotoxin profile of cultured *Prymnesium parvum* (golden algae) and comparing it to samples collected from recent freshwater bloom and fish kill events in North America

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ABSTRACT

Within the last two decades, *Prymnesium parvum* (golden algae) has rapidly spread into inland waterways across the southern portion of North America and this organism has now appeared in more northerly distributed watersheds. In its wake, golden algae blooms have left an alarming trail of ecological devastation, namely massive fish kills, which are threatening the economic and recreational value of freshwater systems throughout the United States. To further understand the nature of this emerging crisis, our group investigated the chemical nature of the toxin(s) produced by *P. parvum*. We approached the problem using a two-pronged strategy that included analyzing both laboratory-grown golden algae and field-collected samples of *P. parvum*. Our results demonstrate that there is a striking difference in the toxin profiles for these two systems. An assemblage of potently ichthyotoxic fatty acids consisting primarily of stearidonic acid was identified in *P. parvum* cultures. While the concentration of the fatty acids alone was sufficient to account for the rapid-onset ichthyotoxic properties of cultured *P. parvum*, we also detected a second type of highly labile ichthyotoxic substance(s) in laboratory-grown golden algae that remains uncharacterized. In contrast, the amounts of stearidonic acid and its related congeners present in samples from recent bloom and fish kill sites fell well below the limits necessary to induce acute toxicity in fish. However, a highly labile ichthyotoxic substance, which is similar to the one found in laboratory-grown *P. parvum* cultures, was also detected. We propose that the uncharacterized labile metabolite produced by *P. parvum* is responsible for golden algae's devastating fish killing effects. Moreover, we have determined that the biologically-relevant ichthyotoxins produced by *P. parvum* are not the prymnesins as is widely believed. Our results suggest that further intensive efforts will be required to chemically define *P. parvum*'s ichthyotoxins under natural bloom conditions.



## 1. Introduction

The marine haptophyte *Prymnesium parvum* (division Haptophyta, class Prymnesiophyceae) (Andersen, 2004), which is commonly referred to as 'golden algae', has played

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causal roles in scores of massive fish kills in coastal marine and high-to-moderate salinity inland waterways throughout the world (Edvardsen and Imai, 2006; Guo et al., 1996; Kaartvedt et al., 1991; Reich and Aschner, 1947; Sabour et al., 2002). In the mid-1980s, the first reported cases documenting the entry of *P. parvum* into North America were marked by multiple large fish kill events in southern Texas, USA (Lopez et al., 2008; Sager et al., 2007). Even more troubling was the realization that *P. parvum* blooms were occurring in moderate-to-low salinity inland lakes, rivers, and reservoirs, which marked a disturbing extension in the range of suitable *P. parvum* habitats (Baker et al., 2009). Consequently, this has caused considerable alarm regarding the ecological and potential human health risks associated with golden algae. Adding to this concern has been the rapid rate of *P. parvum*'s dispersion across the southern United States, and its northerly spread (Aguiar and Kugrens, 2000; Hargraves and Maranda, 2002).

Despite its devastating biological effects, the identity of the toxic constituent(s) produced by *P. parvum* has not been definitively established (Kim and Padilla, 1977; Mariussen et al., 2005; Ulitzur, 1973; Ulitzur and Shilo, 1966). A combination of undefined proteolipids (Ulitzur and Shilo, 1970), ceramides (Wright et al., 2005), saponins (Yariv and Hestrin, 1961), proteinaceous substances (Watson, 2001), plasma membrane/plastid components (Watson, 2001), and proteophospholipids (Watson, 2001) have been credited as responsible for *P. parvum*'s toxicity. In 1982, Kozaki and colleagues proposed that hemolysin I (a combination of galactoglycerolipids) was the *P. parvum* toxin, but no data substantiating the hypothesis were offered (Kozakai et al., 1982). Later in 1996 (Igarashi et al., 1996) and 1999 (Igarashi et al., 1999), Igarashi and colleagues reported the structure determination of the high molecular weight cyclic polyethers prymnesin-1 ($C_{107}H_{154}Cl_3NO_{44}$) (**1**) (Fig. 1) and prymnesin-2 ($C_{96}H_{136}Cl_3NO_{35}$). Both these compounds are potent ichthyotoxins against *Tanichthys albonubes* with $LC_{50}$ values of 8 and 9 nM, respectively (Igarashi et al., 1998). However, none of the prymnesiums have been directly linked to fish kill events and our on-going LC-ESIMS and toxin isolation studies suggest that these compounds do not accumulate at lethal concentrations under laboratory or natural field conditions.

In this study, we used a bioassay-guided approach to identify some of the ichthyotoxic components in laboratory-grown *P. parvum* cultures. These results were compared to LC-ESIMS and GC-EIMS data obtained for two recent fish kill and bloom events caused by golden algae. Given the increasing levels of human contact with *P. parvum* infested waters, extracts and pure compounds were also tested against a human cell line. These data support an important toxic role for several polyunsaturated fatty acids in laboratory-cultured *P. parvum*, but do not fully explain the organism's significant ichthyotoxicity at natural bloom sites.

## 2. Materials and methods

### 2.1. General instrumentation and experimental procedures

HPLC was performed on a Shimadzu preparative instrument using a SCL-10A VP system controller, SPD-10AV VP UV–vis detector, LC-6AD pumps, DGU-14A solvent degasser, and FRC-10A programmable fraction collector. Samples were separated over a Phenomenex C18 Gemini column (5 μm, 110 Å, 250 × 21.2 mm). Semi-preparative HPLC was performed on a similar system using LC-10AT VP pumps and a Phenomenex C18 Gemini column (5 μm, 110 Å, 250 × 10 mm). TOF-ESIMS data were acquired on a Waters LCT Premier instrument. Corrections for exact mass determinations were made automatically with the lock mass feature in the MassLynx software. Samples for mass determination were dissolved in methanol and introduced for ionization using an auto injector with a 20 μL loop. Samples for LC–MS were analyzed by interfacing the HPLC with the ESIMS instrument. GC-EIMS analyses were carried out on a HP 5890 Series II gas chromatograph and a HP 5971A MS detector using a HP 5 (30 m × 0.32 mm × 0.25 μm) cross-linked 5% pH Me siloxane column. Optical rotation measurements were

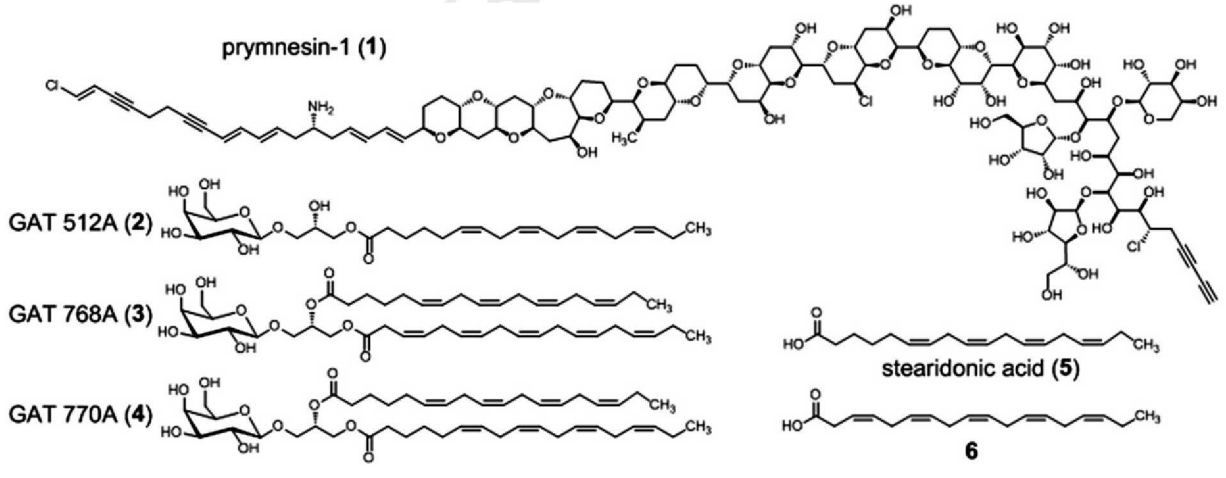


**Fig. 1.** Structures of *P. parvum* derived metabolites including prymnesin-1 (**1**), GAT 512A (**2**), GAT 768A (**3**), GAT 770A (**4**), stearidonic acid (**5**), and (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-3,6,9,12,15-octadecapentanenoic acid (**6**).

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performed on an Autopol III Automatic Polarmeter (Rudolph Research) at 589 nm and 20 °C in water. NMR data were obtained on a Varian VNMR spectrometer at 400 and 500 MHz ($^1$H) and 100 and125 MHz ($^{13}$C), respectively. All solvents were of ACS grade or better.

### 2.2. Biological material

*P. parvum* (UTEX LB2927) was purchased from the University of Texas algal collection. Cultures were grown in 1 L Erlenmeyer flasks containing sterilized COMBO media with added salts (Kilham et al., 1998) and adjusted to pH 7.8. The cultures were capped with vented stoppers and bubbled with filtered (0.22 μm) air. Cultures were maintained under fluorescent grow lights (12 h light/12 h dark photoperiod) until cell densities reached approximately $\sim 2 \times 10^6$ cells/mL as determined with a haemocytometer.

### 2.3. Extraction and isolation

Several sample preparation and extraction techniques were tested to identify the best method for extracting the toxic materials. These methods included extraction of freeze dried culture materials, liquid–liquid partitioning of cultures, and extraction of filtered cells. Ultimately, we determined that liquid–liquid partitioning of the whole cultures (medium plus cells) using ethyl acetate was the best method since the ichthyotoxicity profile of the resulting extract was identical to that of *P. parvum* water both in terms of its potency and rapid-onset toxicity. The ethyl acetate layers from partitioning against 50 L of *P. parvum* cultures were combined and the organic extract (~30 g) was subjected to gradient MPLC over HP20SS resin (30–100% methanol in water with an acetone wash). The fraction eluting with 100% methanol (680 mg) was found to retain all of the ichthyotoxic activity. Preparative reversed-phase HPLC (40–100% acetonitrile in water) yielded a fraction (310 mg) eluting between 75 and 100% acetonitrile that was ichthyotoxic. $^1$H NMR and LC-ESIMS performed on this material showed it contained a variety of polyunsaturated lipids and galactoglycerolipids, but was devoid of any detectable prymnesiums. Fractionation of this material by semi-preparative reversed-phase HPLC consistently resulted in sample sets that quickly lost their ichthyotoxic activities. In addition, many of the $^1$H NMR spins representing the presumed polyunsaturated lipid components showed diminished intensities. After several failed attempts to stabilize the ichthyotoxic substances, we turned our focus toward purifying the three most abundant substances in the fraction. This resulted in the purification (semi-preparative HPLC eluted with 65–85% acetonitrile in water) of compounds **2** (75 mg), **3** (3 mg), and **4** (6 mg).

### 2.4. Enzymatic hydrolysis

For each experiment, a sample consisting of 2–3 mg of galactoglycerolipid was dissolved by sonicating the compound in 2 mL of phosphate buffer (pH 7.0) and then adding a lipase/esterase mixture (Sigma lipase basic kit 62327). The sample was incubated at 30 °C for 36 h after which the reaction mixture was partitioned against dichloromethane. After removal of the solvent from the organic layer *in vacuo*, the remaining residue was resuspended in methanol and immediately analyzed by HPLC for its fatty acid content by comparison of peak retention times to authentic standards. For experiments in which the ichthyotoxicity of hydrolyzed samples were being evaluated, reaction mixtures were not extracted, but instead were directly added to the bioassay vessels containing *Pimephales promelas*. Controls for ichthyotoxicity testing consisted of heat denatured lipase/esterase mixtures.

### 2.5. Alkaline hydrolysis

Compounds (2 mg in 2 mL of methanol) were reacted with 2 mL of 4% (wt./vol.) sodium methoxide in methanol at room temperature for 30 min. Reactions were neutralized by passing over acidic Dowex 50 W X 8 ion-exchange resin. The eluents were partitioned between methanol and hexanes and the methanol-soluble phases were subjected to HPLC separation. $^1$H NMR and optical rotation data ($[\alpha]^{20}_D$ −9.3 (*c* 0.02, water)) were identical to those reported for (2*R*)-3-*O*-[β-D-galactopyranosyl]glycerol (Oshima et al., 2004). The hexanes soluble material was analyzed by GC-EIMS and the presence of methyl stearidonate was confirmed based on comparisons of the samples retention time and EI fragmentation to an authentic standard.

### 2.6. GC-EIMS analysis of fatty acids

The examination of lipids was performed using a method similar to a process described for the transesterification and GC analysis of lipid mixtures (Lepage and Roy, 1986). Laboratory-grown cultures were prepared for fatty acid mixture analysis by lyophilization followed by methanol extraction. After removal of the solvent *in vacuo*, 100 mg samples of the organic extracts were dissolved in 3 mL of methanol–benzene (4:1) and placed in 1 dram vials. Acyl chloride (300 μL aliquots) was added to each vial and the vessels were capped. The mixtures were held at 100 °C for 1 h with constant stirring. Samples were neutralized by adding 6 mL of 6% $K_2CO_3$ to each vial. Samples were briefly sonicated and then centrifuged until phase separation occurred. The upper benzene layers were removed and the samples were directly submitted to GC-EIMS. For the culture-derived samples, the following GC conditions were employed: oven temperature was 100 °C, injection port and transfer lines were held at 250 and 280 °C, respectively. A thermal gradient was applied as follows: held at 100 °C for 3 min followed by a 20 °C/min gradient to 180 °C, a second gradient of 3 °C/min to 225 °C, and a final gradient of 10 °C/min to 250 °C, which was then held for an additional 5 min at 250 °C. Due to the greater complexity of the field-collected samples, we further optimized the GC-EIMS conditions so that samples were held at 100 °C for 3 min followed by a 20 °C/min gradient to a final temperature of 280 °C, which was held for 6 min.

### 2.7. Ichthyotoxicity assay

Determination of the ichthyotoxic properties of *P. parvum* extracts and pure compounds were conducted in accordance



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with EPA-821-R-02-012 (U.S.E.P.A., 2002) (with the minor modifications noted below) and were approved by the University of Oklahoma Institutional Animal Care and Use Committee (IACUC). Briefly, 90 mL clear glass jars were loaded with 50 mL of filtered and aged tap water and 10–14 day old *P. promelas* fry (three per jar) were added and allowed to acclimatize for 1 h. The jars were randomized and samples dissolved in 0.5 mL methanol were added to the jars. Controls consisting of vehicle-only were included in each experiment. Fish were maintained under 12 h light/12 h dark photoperiods at 24 °C. Due to the rapid rate in which the toxin's effects were observed, we monitored fish survivorship at 30 min, 1 h, 24 h and 48 h. We observed that the 1 h mortality counts were typically indistinguishable from observations made at 24 h and 48 h. Each sample was tested in triplicate and the results were expressed as the $LC_{50}$ (the concentration lethal to 50% of fish) ± standard deviation. The $LC_{50}$ values were determined in SigmaPlot v10 (Systat Software Inc) using sigmoidal dose–response regression analyses with variable slope parameters. All fish were euthanized at the conclusion of each experiment.

### 2.8. Mammalian cytotoxicity assay

Evaluations of mammalian cell cytotoxicity were performed as previously described (Mooberry et al., 2007; Tinley et al., 2003). Briefly, cells human (MDA-MB-435 human cancer cell line) were plated in 96-well plates and allowed to adhere and grow for 24 h. Compounds were dissolved in ethanol and cells were incubated with compounds for 48 h. The cells were fixed with TCA and stained with sulforhodamine B. The absorbance was read with a plate reader at 560 nm. Dose–response curves were plotted and the $IC_{50}$ values (the concentration required to inhibit cell proliferation by 50%) were calculated for each experiment. Each sample was tested in triplicate in 3–5 independent experiments and results were expressed as the $IC_{50}$ ± standard deviation.

## 3. Results

### 3.1. Bioassay-guided ichthyotoxin extraction and compound isolation

We have approached the search for *P. parvum* toxins using an ichthyotoxicity-based bioassay-guided methodology. The fathead minnow (*P. promelas*) has served as a reporter organism for detecting biologically-relevant toxins in golden algae cultures and samples collected at two fish kill sites along the Oklahoma–Texas (Lake Texoma – February 2009) and Pennsylvania–West Virginia (Dunkard Creek – October 2009) borders. Our initial studies applying a modified *Kupchan-partitioning* scheme to cultured *P. parvum* demonstrated that golden algae toxins accumulated in the organic layer following water–ethyl acetate partitioning. Bioassays performed on the dried and then reconstituted aqueous layer (resolubilized at ×1, ×10 and ×100 its initial concentration) showed that it was devoid of biologically-relevant ichthyotoxic substances. In contrast, the ×1, ×10 and ×100 reconstituted ethyl acetate layer retained the sample's potent ichthyotoxic properties. Notably, this extraction method varied significantly from the elaborate scheme reported for the isolation of intracellular prymnesins (Igarashi et al., 1999).

The ethyl acetate soluble material collected from 50 L of cultured *P. parvum* was subjected to MPLC (HP20SS resin, gradient elution from 30 to 100% methanol in water followed with a 100% acetone wash). This afforded a single bioactive fraction (100% methanol) that exhibited rapid-onset toxicity against *P. promelas* (note: All fish died within ~20 min when the extract was reconstituted at ×1 its original concentration). We have observed that fish placed in water taken from highly toxic blooms and lab-grown cultures exhibit similar toxicological symptoms that include excessive mucus production near the gills, hyperventilation, and an impaired righting reflex. These symptoms usually appear within 10–20 min of exposure to toxic water and fish typically cease visible movement within 20–30 min. A similar set of symptoms is reportedly brought on by eicosapentaenoic acid (Marshall et al., 2003). The ichthyotoxic sample was subjected to preparative-scale HPLC (reversed-phase octadecyl silica gel, gradient from 40 to 100% acetonitrile in $H_2O$) yielding a single bioactive fraction. The $^1H$ NMR spectrum of the toxic fraction indicated that the sample was composed of several metabolites exhibiting spins spanning the region from $\delta_H$ 0.5 to 5.5, which were strikingly similar to data reported for galactoglycerolipid and polyunsaturated lipid toxins previously isolated from certain unicellular marine organisms (Fu et al., 2004; Hiraga et al., 2002; Kobayashi et al., 1992; Stabell et al., 1993) including *P. parvum* (Kozakai et al., 1982). Subsequent semi-preparative reversed-phase HPLC performed on a portion of the bioactive sample yielded a series of fractions that exhibited greatly diminished toxicity, which was spread across several consecutive fractions. However, even after recombining the samples, the ichthyotoxic properties of the mixed fractions were substantially reduced (>10-fold loss of toxicity) and all samples were devoid of activity following brief storage (dried and held for <24 h at 4 °C). Returning our attention to the parent preparative HPLC fraction, we found that it still retained toxicity and so we set about characterizing the sample's three major components.

Positive-ion HRESIMS of the purified metabolites provided quasi-molecular ions $[M + Na]^+$ at *m/z* 535.2842 (calcd for $C_{27}H_{44}NaO_9$, 535.2883), 791.4678 (calcd for $C_{45}H_{68}NaO_{10}$, 791.4710), and 793.4801 (calcd for $C_{45}H_{70}NaO_{10}$, 793.4867) for metabolites **2** (GAT 512A), **3** (GAT 768A), and **4** (GAT 770A), respectively (Fig. 1). In order to provide a simple naming scheme for compounds **2**, **3**, and **4**, we have proposed the use of the term "GATs" (golden algae toxins) rather than the more lengthy IUPAC nomenclature. Each GAT is assigned a unique identifier number based on its molecular weight, which is followed by a letter code to distinguish isomers from each other. Accordingly, compounds **2**, **3**, and **4** have been termed GAT 512A, GAT 768A, and GAT 770A, respectively. Further data obtained from 1D- ($^1H$ and $^{13}C$ NMR) and 2D- (COSY, HSQC, and HMBC) NMR (Supplementary Information, Table 1), controlled chemical and enzymatic degradation experiments, and optical rotation measurements of the metabolites and their respective hydrolysis products allowed us to



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**Table 1**
Determination of the cell density, stearidonic acid concentrations, and ichthyotoxicity of golden algae cultures and water samples collected at recent *P. parvum* associated fish kill and bloom events.

| Sample | *P. parvum* cells density ($\times 10^5$ cells/mL ± SD) | Stearidonic acid (**5**) content (μM) | Ichthyotoxic (yes/no) |
|---|---|---|---|
| 7 Day old laboratory culture | 8.2 ± 0.7 | 5.8 ± 1.8 | No |
| 12 Day old laboratory culture | 13.0 ± 0.4 | 31.2 ± 7.7 | Yes |
| 20 Day old laboratory culture | 24.0 ± 3.0 | 95.2 ± 23.7 | Yes |
| Bloom event in Lake Texoma, Lebanon Pool (Feb. 2009) | 1.2 ± 0.1 | 0.06 ± 0.02 | Yes |
| Post bloom in Lake Texoma, Lebanon Pool (June 2009) | Not detected | Not detected | No |
| Fish kill event in Dunkard Creek drainage, Wana Bridge (Oct. 2009) | 5.9 ± 1.5 | 0.44 ± 0.16 | Yes |
| Non-fish kill area in Dunkard Creek drainage, Upper Beaver Dam (Oct. 2009) | Not detected | Not detected | No |

confirm the structures of these compounds. The identities of these compounds were also confirmed by comparisons of our data with values published in the literature for **2** (Hiraga et al., 2008), **3** (Kobayashi et al., 1992), and **4** (Kobayashi et al., 1992). Surprisingly, **2**–**4** exhibited no ichthyotoxicity against *P. promelas* (up to 430 μM); however, we did observe modest cytotoxicity for **2** in a human (MDA-MB-435) cancer cell line ($IC_{50}$ 24.2 ± 5.1 μM).

### 3.2. Esterase mediated liberation of ichthyotoxic fatty acids

Further consideration of structures **2**–**4** suggested to us that GATs might function as protoxins since the polyunsaturated *O*-alkyl esters moieties found within these metabolites could be readily hydrolyzed resulting in the release of their corresponding carboxylic acid derivatives. Similar metabolites have been implicated as potent toxins against fish (Marshall et al., 2003) and isolated fish cell lines (Fossat et al., 1999). We hypothesized that esterases, which are ubiquitous hydrolytic enzymes found in aquatic ecosystems (Mudryk and Skórczewski, 2006), could readily catalyze the release of polyunsaturated fatty acids from the **2**–**4** (Fig. 2). We tested this by incubating **2**–**4** with assorted esterases and this resulted in the liberation of a potent mixture of ichthyotoxins that included stearidonic acid (**5**) and its analog **6** (Fig. 1). None of the controls utilizing heat denatured esterases showed any ichthyotoxic properties (Fig. 2). More importantly, tests performed using purified and commercially available **5** confirmed that the concentration of this fatty acid alone was sufficient to cause rapid-onset toxicity ($LC_{50}$ 21.9 ± 6.3 μM; all fish dead in <20 min at 40 μM) and other distinct pathological features that are typically observed when *P. promelas* is exposed to *P. parvum* cultures.

### 3.3. Bioassay analysis of fatty acids

We expanded our testing of **5** to include several of its derivatives (*i.e.*, methyl steridonate, steridonoyl glycine, and stearic acid); however, none of these compounds were toxic to fish at concentrations of ≤40 μM (Fig. 3). An examination of 16 additional lipids led to the identification of several other fatty acids that displayed similar or increased potency against fish (docosahexanoeic acid: $LC_{50}$ 4.7 ± 1.3 μM; eicosapentaenoic acid: $LC_{50}$ 23.6 ± 9.0 μM, arachindonic acid: $LC_{50}$ 9.2 ± 0.8 μM, and pinolenic acid: $LC_{50}$ 18.2 ± 5.9 μM) (Fig. 3). These results suggest that a combination of a carboxylic acid and ≥2 double bonds in a *Z* configuration are necessary to confer ichthyotoxic properties to fatty acids. Given the modest size of our lipid library, further speculation regarding the structural features required to impart ichthyotoxic properties to fatty acids is not possible at this time. The cytotoxicity of the lipids was also tested against human cells (MDA-MB-435); however, none of the compounds inhibited cell proliferation at concentrations ≤10 μM.

### 3.4. GC-EIMS assessment of cultured P. parvum lipids and comparisons to water from bloom and fish kill sites

Tests performed by GC-EIMS on laboratory-grown *P. parvum* enabled us to determine that this organism produces a complex suite of lipophilic metabolites. A representative GC-EIMS trace showing the lipid profile of a 20 day old *P. parvum* culture is provided in Supplemental Information, Fig. 1A. The bulk of this mixture was found to consist of a combination of saturated and unsaturated $C_{16}$, $C_{18}$, $C_{20}$, and $C_{22}$ compounds. In view of the toxicity data obtained from tests performed with our lipid library (Fig. 3), we conjecture that in addition to **5**, some of the other polyunsaturated $C_{20}$ and $C_{22}$ metabolites might contribute to the ichthyotoxicity profile of cultured *P. parvum*.

We also assessed the quantity of **5** in cultured *P. parvum* since this compound represented one of the most prevalent (second only to myristic acid) substances in the mixture and we were able to secure authentic samples for analytical comparisons. A seemingly positive correlation was observed between *P. parvum* cell numbers, the concentration of **5** in cultures, and toxicity of culture water against *P. promelas* (Table 1). Specifically, we noted that as the cell densities of *P. parvum* cultures approached $10^6$ cells/mL, the levels of **5** significantly exceeded the compound's $LC_{50}$ value (Table 1). Next, we compared the results from cultured golden algae with data from field-collected water samples obtained from recent fish kill/bloom sites. We immediately discerned two substantial differences between laboratory-grown golden algae and field samples taken from recent blooms: 1) the cell density for wild *P.*



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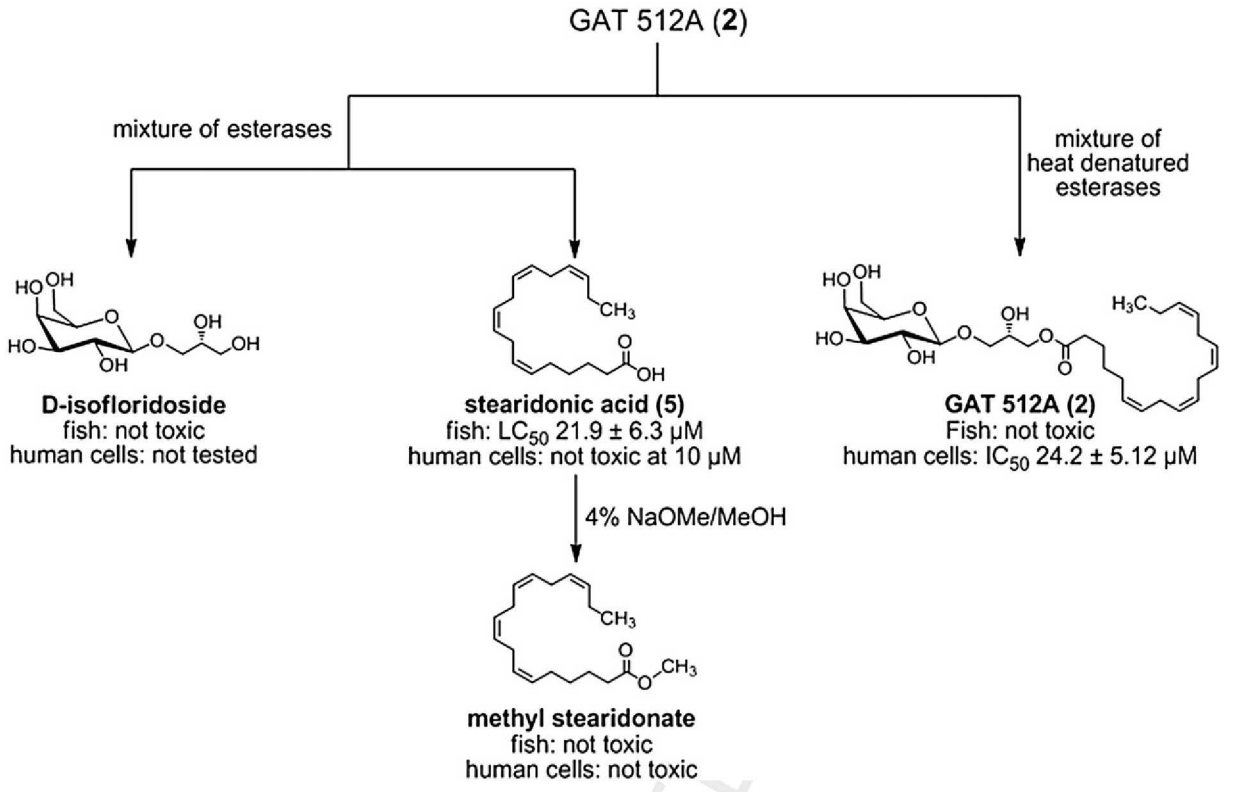


**Fig. 2.** Assessment of the ichthyotoxicity and mammalian cell cytotoxicity of compounds obtained from the enzymatic hydrolysis of compound **2**. Incubation of **2** with a lipase/esterase mixture resulted in the liberation of D-isofloridoside and **5**, which was ichthyotoxic. Conversion of **5** to its methyl ester derivative **6** resulted in the complete loss of ichthyotoxicity. Incubation of **2** with a heat denatured lipase/esterase mixture yielded unconverted **2**, which was not toxic to fish, but did exhibit modest cytotoxicity toward mammalian cells.

*parvum* populations did not reach the same high levels achieved in laboratory cultures and 2) the concentration of **5** was proportionally reduced. While our analysis of field-collected samples did confirm the presence of **5** at bloom and fish kill events (Supplemental Information, Fig. 1B and C), its concentrations during these periods failed to reach toxic levels (<0.5 μM) (Table 1). Interestingly, we did not detect **5** at nearby non-bloom sites or in water gathered during non-bloom periods (Table 1 and Supplemental Information, Fig. 1D). This suggests that **5** may serve as a chemical marker for impending or on-going *P. parvum* blooms.

## 4. Discussion

Despite our many attempts using LC-ESIMS, direct injection ESIMS (positive and negative modes), and bioassay-guided approaches to detect prymnesins (*e.g.*, compound **1**), we were unable to generate conclusive evidence that these cyclic polyethers meaningfully contribute to the ichthyotoxic properties of laboratory-grown or field samples *P. parvum*. Instead, a suite of uncommon polyunsaturated fatty acids and their conjugated galactoglycerolipid progenitors have emerged as important chemical agents that appreciably contribute to the ichthyotoxic effects of cultured *P. parvum*. This finding is supported by a substantial body of anecdotal evidence and extensive observations concerning the seemingly mixed lipophilic and amphiphilic properties of semi-purified and purified *P. parvum* toxins (Shilo, 1971; Ulitzur, 1973; Ulitzur and Shilo, 1970; Yariv and Hestrin, 1961). Moreover, the labile nature of the *P. parvum*'s toxins can be partially explained based on numerous autooxidative degradation processes that polyunsaturated fatty acids can undergo (Schauenstein, 1967; VanRollins and Murphy, 1984). Even if other ichthyotoxic compounds are biosynthesized by laboratory-grown *P. parvum*, the quantity of toxic fatty acids alone is sufficient to render the cultures lethal to fish. Although we are unaware of considerable human health risks attributable to dermal contact, inhalation, or ingestion of galactoglycerolipids and polyunsaturated lipids such as **2**–**6**, we recommend that caution be used while handling concentrated samples of these substances until further toxicological risk assessment studies have been conducted.

We are confident that prymnesins are not this organism's primary toxin. This assertion is based on several key observations including: 1) prymnesins were not detected by LC-ESIMS in cultured or field-collected water samples where high *P. parvum* cell densities occurred, 2) bioassay-guided isolation methods have not provided any fractions in which $^{1}$H NMR or ESIMS have yielded unequivocal evidence for prymnesins or prymnesin-degradation products, 3) the labile nature of the *P. parvum* toxin is an



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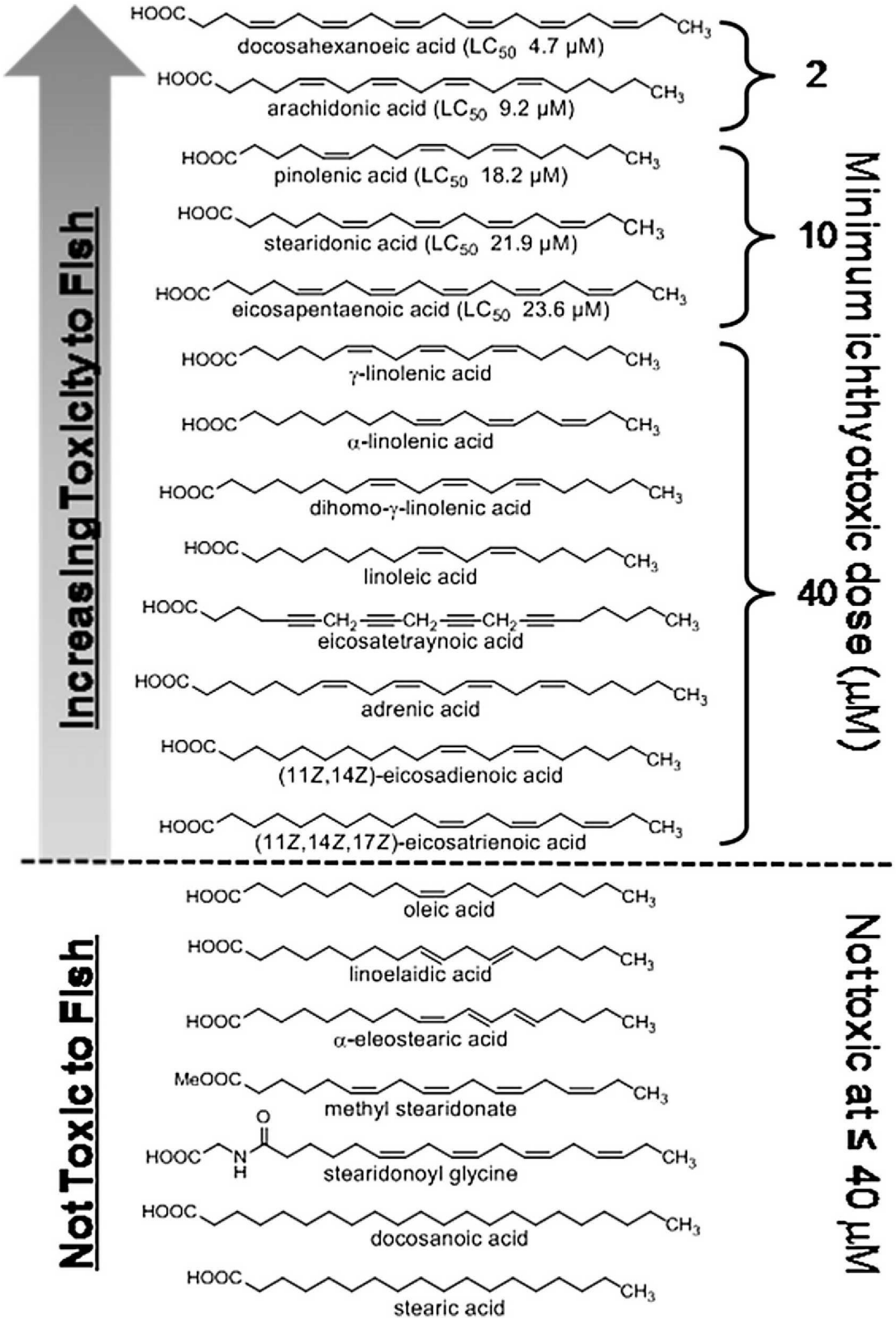


**Fig. 3.** Library of lipids tested for ichthyotoxic properties. Compounds are arranged based on their relative potencies against *P. promelas* fry.

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unlikely characteristic of prymnesins given their described structures and lack of apparent instabilities. While preliminary FABMS data hint at the presence of high molecular weight (prymnesin-like) compounds in certain *P. parvum* samples (weak quasi-molecular ions have been observed between *m/z* 2100 and 2500; data not shown), we have not been able to link the occurrence of these substances with ichthyotoxic effects. Moreover, the quantities of these putative high molecular weight metabolites fall well below their reported $LC_{50}$ values (*e.g.*, the detection limits for FABMS are generally within the low nanogram to picogram range; the weak intensities of the peaks we have observed would put the concentrations of these compounds at levels that are $\geq$10–100-fold below their $LC_{50}$ values). Instead, our data support the idea that golden algae produce another, yet uncharacterized ichthyotoxin(s).

Our results are particularly significant in view of the increasing frequency with which people are becoming exposed to *P. parvum* infested waters and the current lack of concern regarding human contact with this organism (TPWD, 2007; AGFD, 2009). Although our data provide convincing evidence for certain fatty acids serving as the predominant toxins in laboratory-grown *P. parvum* cultures, we have not yet determined the identity of the ichthyotoxic compound(s) responsible for the growing number of fish kill events in the United States. However, we cannot rule out the possibility that toxic fatty acids may exhibit ichthyotoxic effects when administered chronically to fish at low-dosages. Given the different growth conditions experienced by *P. parvum* in the laboratory versus in nature, it is probable that golden algae produce different toxins under these different conditions. We will continue to investigate methods for preserving the integrity of the labile *P. parvum* toxin (*e.g.*, consideration of pH (Valenti et al., 2009), free radicals (Marshall et al., 2003), and possible metal interactions (Moeller et al., 2007)) and we hope to report these findings in due course.

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## Appendix: Supplemental information

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.toxicon.2010.02.017.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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